Dkt. 60795/JPW

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that I,

Marie Sudam Pathirana

have invented certain new and useful improvements in

DNA ENCODING ORPHAN SNORF68 RECEPTOR

of which the following is a full, clear and exact description.

DNA ENCODING ORPHAN SNORF68 RECEPTOR

BACKGROUND OF THE INVENTION

Throughout this application various publications are referred to by partial citations within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which the invention pertains.

Neuroregulators comprise a diverse group of natural products that subserve or modulate communication in the nervous system. They include, but are not limited to, neuropeptides, amino acids, biogenic amines, lipids and lipid metabolites, and other metabolic byproducts. Many of these neuroregulator substances interact with specific cell surface receptors which transduce signals from the outside to the inside of the cell. G-protein coupled receptors (GPCRs) represent a major class of cell surface receptors with which many neurotransmitters interact to mediate their effects. GPCRs are characterized by seven membrane-spanning domains and are coupled to their effectors via G-proteins linking receptor activation with intracellular biochemical sequelae such as stimulation of adenylyl cyclase. While the structural motifs that characterize a GPCR can be recognized in the predicted amino acid sequence of a novel receptor, the endogenous ligand that activates the GPCR cannot necessarily be predicted from its primary structure. Thus, a novel receptor sequence

may be designated as an orphan GPCR when it possesses the structural motif characteristic of a G-protein coupled receptor, but its endogenous ligand has not yet been defined.

SUMMARY OF THE INVENTION

This invention provides a recombinant nucleic acid comprising a nucleic acid encoding a mammalian SNORF68 receptor, wherein the mammalian receptor-encoding nucleic acid hybridizes under high stringency conditions to a nucleic acid encoding a human SNORF68 receptor and having a sequence identical to the sequence of the human SNORF68 receptor-encoding nucleic acid contained in plasmid pEXJ.T3T7-hSNORF68-f (Patent Deposit Designation No. PTA_____).

This invention further provides a recombinant nucleic acid comprising a nucleic acid encoding a human SNORF68 receptor, wherein the human SNORF68 receptor comprises an amino acid sequence identical to the sequence of the human SNORF68 receptor encoded by the shortest open reading frame indicated in Figures 1A-1C (SEQ ID NO: 1).

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C

Nucleotide sequence including sequence encoding a human SNORF68 receptor (SEQ ID NO: 1). Putative open reading frames including the shortest open reading frame are indicated by underlining two start (ATG) codons (at positions 26-28 and 62-64) and the stop codon (at positions 1508-1510). In addition, partial 5' and 3' untranslated sequences are shown.

Figures 2A-2C

Deduced amino acid sequence (SEQ ID NO: 2) of the human SNORF68 receptor encoded by the longest open reading frame indicated in the nucleotide sequence shown in Figures 1A-1C (SEQ ID NO: 1). The seven putative transmembrane (TM) regions are underlined.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a recombinant nucleic acid comprising a nucleic acid encoding a mammalian SNORF68 receptor, wherein the mammalian receptor-encoding nucleic acid hybridizes under high stringency conditions to a nucleic acid encoding a human SNORF68 receptor and having a sequence identical to the sequence of the human SNORF68 receptor-encoding nucleic acid contained in plasmid pEXJ.T3T7-hSNORF68-f (Patent Deposit Designation No. PTA_____).

This invention further provides a recombinant nucleic acid comprising a nucleic acid encoding a human SNORF68 receptor, wherein the human SNORF68 receptor comprises an amino acid sequence identical to the sequence of the human SNORF68 receptor encoded by the shortest open reading frame indicated in Figures 1A-1C (SEQ ID NO: 1).

This invention also contemplates recombinant nucleic acids which comprise nucleic acids encoding naturally occurring allelic variants of the above.

The plasmid pEXJ.T3T7-hSNORF68-f was deposited on _____, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded Patent Deposit Designation No. PTA_____.

Hybridization methods are well known to those of

skill in the art. For purposes of this invention, hybridization under high stringency conditions means hybridization performed at 40°C in a hybridization buffer containing 50% formamide, 5X SSC, 7 mM Tris, 1X Denhardt's, 25 µg/ml salmon sperm DNA; wash at 50°C in 0.1X SSC, 0.1% SDS.

The nucleic acids of this invention may be used as probes to obtain homologous nucleic acids from other species and to detect the existence of nucleic acids having complementary sequences in samples.

The nucleic acids may also be used to express the receptors they encode in transfected cells.

Also, use of the receptor encoded by the SNORF68 receptor nucleic acid sequence enables the discovery of the endogenous ligand.

The use of a constitutively active receptor encoded by SNORF68 either occurring naturally without further modification or after appropriate point mutations, deletions or the like, allows screening for antagonists and *in vivo* use of such antagonists to attribute a role to receptor SNORF68 without prior knowledge of the endogenous ligand.

Use of the nucleic acids further enables elucidation of possible receptor diversity and of the existence of multiple subtypes within a family of receptors of which SNORF68 is a member.

Finally, it is contemplated that this receptor will serve as a valuable tool for designing drugs for treating various pathophysiological conditions such

as chronic and acute inflammation, arthritis, autoimmune diseases, transplant rejection, graft vs. host disease, bacterial, fungal, protozoan and viral infections, septicemia, AIDS, pain, psychotic and neurological disorders, including anxiety, depression, schizophrenia, dementia, mental retardation, memory loss, epilepsy, locomotor problems, respiratory disorders, asthma, eating/body weight disorders including obesity, bulimia, diabetes, anorexia, nausea, hypertension, hypotension, vascular and cardiovascular disorders, ischemia, stroke, cancers, ulcers, urinary retention, sexual/reproductive disorders, circadian rhythm disorders, renal disorders, bone diseases including osteoporosis, benign prostatic hypertrophy, gastrointestinal disorders, nasal congestion, allergies, Parkinson's disease, Alzheimer's disease, among others and diagnostic assays for such conditions.

Methods of transfecting cells e.g. mammalian cells, with such nucleic acid to obtain cells in which the receptor is expressed on the surface of the cell are well known in the art. (See, for example, U.S. Patent Nos. 5,053,337; 5,155,218; 5,360,735; 5,472,866; 5,476,782; 5,516,653; 5,545,549; 5,556,753; 5,595,880; 5,602,024; 5,639,652; 5,652,113; 5,661,024; 5,766,879; 5,786,155; and 5,786,157, the disclosures of which are hereby incorporated by reference in their entireties into this application.)

Such transfected cells may also be used to test compounds and screen compound libraries to obtain compounds which bind to the orphan SNORF68 receptor,

as well as compounds which activate or inhibit activation of functional responses in such cells, and therefore are likely to do so in vivo. (See, for example, U.S. Patent Nos. 5,053,337; 5,155,218; 5,360,735; 5,472,866; 5,476,782; 5,516,653; 5,545,549; 5,556,753; 5,595,880; 5,602,024; 5,639,652; 5,652,113; 5,661,024; 5,766,879; 5,786,155; and 5,786,157, the disclosures of which are hereby incorporated by reference in their entireties into this application.)

Host cells

A broad variety of host cells can be used to study heterologously expressed proteins. These cells include but are not limited to mammalian cell lines such as; Cos-7, CHO, LM(*tk*$^-$), HEK293, etc.; insect cell lines such as; Sf9, Sf21, etc.; amphibian cells such as *xenopus oocytes*; assorted yeast strains; assorted bacterial cell strains; and others. Culture conditions for each of these cell types is specific and is known to those familiar with the art.

Transient expression

DNA encoding proteins to be studied can be transiently expressed in a variety of mammalian, insect, amphibian, yeast, bacterial and other cells lines by several transfection methods including but not limited to; calcium phosphate-mediated, DEAE-dextran mediated; liposomal-mediated, viral-mediated, electroporation-mediated, and microinjection delivery. Each of these methods may require optimization of assorted experimental parameters depending on the DNA, cell line, and the type of assay to be subsequently employed.

Stable expression

Heterologous DNA can be stably incorporated into host cells, causing the cell to perpetually express a foreign protein. Methods for the delivery of the DNA into the cell are similar to those described above for transient expression but require the co-transfection of an ancillary gene to confer drug resistance on the targeted host cell. The ensuing drug resistance can be exploited to select and maintain cells that have taken up the DNA. An assortment of resistance genes are available including but not restricted to neomycin, kanamycin, and hygromycin. For the purposes of studies concerning the orphan receptor of this invention, stable expression of a heterologous receptor protein is typically carried out in, mammalian cells including but not necessarily restricted to, CHO, HEK293, LM(tk-), etc.

In addition native cell lines that naturally carry and express the nucleic acid sequences for the orphan receptor may be used without the need to engineer the receptor complement.

Membrane preparations

Cell membranes expressing the orphan receptor protein of this invention are useful for certain types of assays including but not restricted to ligand binding assays, GTP-γ-S binding assays, and others. The specifics of preparing such cell membranes may in some cases be determined by the nature of the ensuing assay but typically involve harvesting whole cells and disrupting the cell pellet by sonication in ice cold buffer (e.g. 20 mM Tris-HCl, 5 mM EDTA, pH 7.4). The resulting crude cell lysate is cleared of cell

debris by low speed centrifugation at 200xg for 5 min at 4°C. The cleared supernatant is then centrifuged at 40,000xg for 20 min at 4°C, and the resulting membrane pellet is washed by suspending in ice cold buffer and repeating the high speed centrifugation step. The final washed membrane pellet is resuspended in assay buffer. Protein concentrations are determined by the method of Bradford (1976) using bovine serum albumin as a standard. The membranes may be used immediately or frozen for later use.

Generation of baculovirus

The coding region of DNA encoding the human receptor disclosed herein may be subcloned into pBlueBacIII into existing restriction sites or sites engineered into sequences 5' and 3' to the coding region of the polypeptides. To generate baculovirus, 0.5 µg of viral DNA (BaculoGold) and 3 µg of DNA construct encoding a polypeptide may be co-transfected into 2 x 10^6 *Spodoptera frugiperda* insect Sf9 cells by the calcium phosphate co-precipitation method, as outlined by Pharmingen (in "Baculovirus Expression Vector System: Procedures and Methods Manual"). The cells then are incubated for 5 days at 27°C.

The supernatant of the co-transfection plate may be collected by centrifugation and the recombinant virus plaque purified. The procedure to infect cells with virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

Labeled ligand binding assays

Cells expressing the orphan receptor of this invention may be used to screen for ligands for said receptors, for example, by labeled ligand binding

assays. Once a ligand is identified the same assays may be used to identify agonists or antagonists of the orphan receptor that may be employed for a variety of therapeutic purposes.

In an embodiment, labeled ligands are placed in contact with either membrane preparations or intact cells expressing the orphan receptor in multi-well microtiter plates, together with unlabeled compounds, and binding buffer. Binding reaction mixtures are incubated for times and temperatures determined to be optimal in separate equilibrium binding assays. The reaction is stopped by filtration through GF/B filters, using a cell harvester, or by directly measuring the bound ligand. If the ligand was labeled with a radioactive isotope such as ^{3}H, ^{14}C, ^{125}I, ^{35}S, ^{32}P, ^{33}P, etc., the bound ligand may be detected by using liquid scintillation counting, scintillation proximity, or any other method of detection for radioactive isotopes. If the ligand was labeled with a fluorescent compound, the bound labeled ligand may be measured by methods such as, but not restricted to, fluorescence intensity, time resolved fluorescence, fluorescence polarization, fluorescence transfer, or fluorescence correlation spectroscopy. In this manner agonist or antagonist compounds that bind to the orphan receptor may be identified as they inhibit the binding of the labeled ligand to the membrane protein or intact cells expressing the said receptor. Non-specific binding is defined as the amount of labeled ligand remaining after incubation of membrane protein in the presence of a high concentration (e.g., 100-1000 X K_D) of unlabeled ligand. In equilibrium saturation binding assays membrane preparations or intact cells

transfected with the orphan receptor are incubated in the presence of increasing concentrations of the labeled compound to determine the binding affinity of the labeled ligand. The binding affinities of unlabeled compounds may be determined in equilibrium competition binding assays, using a fixed concentration of labeled compound in the presence of varying concentrations of the displacing ligands.

Functional assays

Cells expressing the orphan receptor DNA of this invention may be used to screen for ligands to said receptor using functional assays. Once a ligand is identified the same assays may be used to identify agonists or antagonists of the orphan receptor that may be employed for a variety of therapeutic purposes. It is well known to those in the art that the over-expression of a G-protein coupled receptor can result in the constitutive activation of intracellular signaling pathways. In the same manner, over-expression of the orphan receptor in any cell line as described above, can result in the activation of the functional responses described below, and any of the assays herein described can be used to screen for both agonist and antagonist ligands of the orphan receptor.

A wide spectrum of assays can be employed to screen for the presence of orphan receptor ligands. These assays range from traditional measurements of total inositol phosphate accumulation, cAMP levels, intracellular calcium mobilization, and potassium currents, for example; to systems measuring these same second messengers but which have been modified or adapted to be of higher throughput, more generic

and more sensitive; to cell based assays reporting more general cellular events resulting from receptor activation such as metabolic changes, differentiation, cell division/proliferation. Description of several such assays follow.

Cyclic AMP (cAMP) assay

The receptor-mediated stimulation or inhibition of cyclic AMP (cAMP) formation may be assayed in cells expressing the receptors. Cells are plated in 96-well plates or other vessels and preincubated in a buffer such as HEPES buffered saline (NaCl (150 mM), $CaCl_2$ (1 mM), KCl (5 mM), glucose (10 mM)) supplemented with a phosphodiesterase inhibitor such as 5mM theophylline, with or without protease inhibitor cocktail (For example, a typical inhibitor cocktail contains 2 µg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 µg/ml phosphoramidon.) for 20 min at 37°C, in 5% CO_2. Test compounds are added with or without 10 mM forskolin and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl or other methods. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution is measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software. Specific modifications may be performed to optimize the assay for the orphan receptor or to alter the detection method of cAMP.

Arachidonic acid release assay

Cells expressing the orphan receptor are seeded into 96 well plates or other vessels and grown for 3 days in medium with supplements. ^{3}H-arachidonic acid

(specific activity = 0.75 µCi/ml) is delivered as a 100 µL aliquot to each well and samples are incubated at 37° C, 5% CO_2 for 18 hours. The labeled cells are washed three times with medium. The wells are then filled with medium and the assay is initiated with the addition of test compounds or buffer in a total volume of 250 µL. Cells are incubated for 30 min at 37°C, 5% CO_2. Supernatants are transferred to a microtiter plate and evaporated to dryness at 75°C in a vacuum oven. Samples are then dissolved and resuspended in 25 µL distilled water. Scintillant (300 µL) is added to each well and samples are counted for ^{3}H in a Trilux plate reader. Data are analyzed using nonlinear regression and statistical techniques available in the GraphPAD Prism package (San Diego, CA).

<u>Intracellular calcium mobilization assays</u>

The intracellular free calcium concentration may be measured by microspectrofluorimetry using the fluorescent indicator dye Fura-2/AM (Bush et al, 1991). Cells expressing the receptor are seeded onto a 35 mm culture dish containing a glass coverslip insert and allowed to adhere overnight. Cells are then washed with HBS and loaded with 100 µL of Fura-2/AM (10 µM) for 20 to 40 min. After washing with HBS to remove the Fura-2/AM solution, cells are equilibrated in HBS for 10 to 20 min. Cells are then visualized under the 40X objective of a Leitz Fluovert FS microscope and fluorescence emission is determined at 510 nM with excitation wavelengths alternating between 340 nM and 380 nM. Raw fluorescence data are converted to calcium concentrations using standard calcium concentration curves and software analysis techniques.

In another method, the measurement of intracellular calcium can also be performed on a 96-well (or higher) format and with alternative calcium-sensitive indicators, preferred examples of these are: aequorin, Fluo-3, Fluo-4, Fluo-5, Calcium Green-1, Oregon Green, and 488 BAPTA. After activation of the receptors with agonist ligands the emission elicited by the change of intracellular calcium concentration can be measured by a luminometer, or a fluorescence imager; a preferred example of this is the fluorescence imager plate reader (FLIPR).

Cells expressing the receptor of interest are plated into clear, flat-bottom, black-wall 96-well plates (Costar) at a density of 30,000-80,000 cells per well and allowed to incubate over night at 5% CO_2, 37°C. The growth medium is aspirated and 100 µl of dye loading medium is added to each well. The loading medium contains: Hank's BSS (without phenol red)(Gibco), 20 mM HEPES (Sigma), 0.1% BSA (Sigma), dye/pluronic acid mixture (e.g. 1 mM Flou-3, AM (Molecular Probes), 10% pluronic acid (Molecular Probes); (mixed immediately before use), and 2.5 mM probenecid (Sigma)(prepared fresh)). The cells are allowed to incubate for about 1 hour at 5% CO_2, 37°C.

During the dye loading incubation the compound plate is prepared. The compounds are diluted in wash buffer (Hank's BSS without phenol red), 20 mM HEPES, 2.5 mM probenecid to a 3X final concentration and aliquoted into a clear v-bottom plate (Nunc). Following the incubation the cells are washed to remove the excess dye. A Denley plate washer is used to gently wash the cells 4 times and leave a 100 µl final volume of wash buffer in each well. The cell

plate is placed in the center tray and the compound plate is placed in the right tray of the FLIPR. The FLIPR software is setup for the experiment, the experiment is run and the data are collected. The data are then analyzed using an excel spreadsheet program.

Antagonist ligands are identified by the inhibition of the signal elicited by agonist ligands.

Inositol phosphate assay

Receptor mediated activation of the inositol phosphate (IP) second messenger pathways may be assessed by radiometric or other measurement of IP products.

For example, in a 96 well microplate format assay, cells are plated at a density of 70,000 cells per well and allowed to incubate for 24 hours. The cells are then labeled with 0.5 µCi [^{3}H]myo-inositol overnight at 37^{0}C, 5% CO_2. Immediately before the assay, the medium is removed and replaced with 90 µL of PBS containing 10 mM LiCl. The plates are then incubated for 15 min at 37^{0}C, 5% CO_2. Following the incubation, the cells are challenged with agonist (10 µl/well; 10x concentration) for 30 min at 37^{0}C, 5% CO_2. The challenge is terminated by the addition of 100 µL of 50% v/v trichloroacetic acid, followed by incubation at 4^{0}C for greater than 30 minutes. Total IPs are isolated from the lysate by ion exchange chromatography. Briefly, the lysed contents of the wells are transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates are prepared adding 100 µL of Dowex AG1-X8 suspension (50% v/v,

water: resin) to each well. The filter plates are placed on a vacuum manifold to wash or elute the resin bed. Each well is first washed 2 times with 200 µl of 5 mM myo-inositol. Total [^{3}H]inositol phosphates are eluted with 75 µl of 1.2M ammonium formate/0.1M formic acid solution into 96-well plates. 200 µL of scintillation cocktail is added to each well, and the radioactivity is determined by liquid scintillation counting.

<u>GTPγS functional assay</u>

Membranes from cells expressing the orphan receptor are suspended in assay buffer (e.g., 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 10 µM GDP, pH 7.4) with or without protease inhibitors (e.g., 0.1% bacitracin). Membranes are incubated on ice for 20 minutes, transferred to a 96-well Millipore microtiter GF/C filter plate and mixed with GTPγ^{35}S (e.g., 250,000 cpm/sample, specific activity ~1000 Ci/mmol) plus or minus unlabeled GTPγS (final concentration = 100 µM). Final membrane protein concentration ≈ 90 µg/ml. Samples are incubated in the presence or absence of test compounds for 30 min. at room temperature, then filtered on a Millipore vacuum manifold and washed three times with cold (4^{0}C) assay buffer. Samples collected in the filter plate are treated with scintillant and counted for ^{35}S in a Trilux (Wallac) liquid scintillation counter. It is expected that optimal results are obtained when the receptor membrane preparation is derived from an appropriately engineered heterologous expression system, i.e., an expression system resulting in high levels of expression of the receptor and/or expressing G-proteins having high turnover rates (for the exchange of GDP for GTP). GTPγS assays are well-

known to those skilled in the art, and it is contemplated that variations on the method described above, such as are described by Tian et al. (1994) or Lazareno and Birdsall (1993), may be used.

Microphysiometric assay

Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any orphan receptor regardless of the specifics of the receptor's signaling pathway.

General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Typically cells expressing receptors are harvested and seeded at 3 x 10^5 cells per microphysiometer capsule in complete media 24 hours prior to an experiment. The media is replaced with serum free media 16 hours prior to recording to minimize non-specific metabolic stimulation by assorted and ill-defined serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established.

A standard recording protocol specifies a 100 µl/min flow rate, with a 2 min total pump cycle which

includes a 30 sec flow interruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10 μM final concentration. Follow up experiments to examine dose-dependency of active compounds are then done by sequentially challenging the cells with a drug concentration range that exceeds the amount needed to generate responses ranging from threshold to maximal levels. Ligand samples are then washed out and the acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

<u>MAP kinase assay</u>

MAP kinase (mitogen activated kinase) may be monitored to evaluate receptor activation. MAP kinase is activated by multiple pathways in the cell. A primary mode of activation involves the ras/raf/MEK/MAP kinase pathway. Growth factor (tyrosine kinase) receptors feed into this pathway via SHC/Grb-2/SOS/ras. Gi coupled receptors are also known to activate ras and subsequently produce an activation of MAP kinase. Receptors that activate phospholipase C (such as Gq/G11-coupled) produce diacylglycerol (DAG) as a consequence of phosphatidyl inositol hydrolysis. DAG activates protein kinase C which in turn phosphorylates MAP kinase.

MAP kinase activation can be detected by several approaches. One approach is based on an evaluation

of the phosphorylation state, either unphosphorylated (inactive) or phosphorylated (active). The phosphorylated protein has a slower mobility in SDS-PAGE and can therefore be compared with the unstimulated protein using Western blotting. Alternatively, antibodies specific for the phosphorylated protein are available (New England Biolabs) which can be used to detect an increase in the phosphorylated kinase. In either method, cells are stimulated with the test compound and then extracted with Laemmli buffer. The soluble fraction is applied to an SDS-PAGE gel and proteins are transferred electrophoretically to nitrocellulose or Immobilon. Immunoreactive bands are detected by standard Western blotting technique. Visible or chemiluminescent signals are recorded on film and may be quantified by densitometry.

Another approach is based on evaluation of the MAP kinase activity via a phosphorylation assay. Cells are stimulated with the test compound and a soluble extract is prepared. The extract is incubated at 30°C for 10 min with gamma-^{32}P-ATP, an ATP regenerating system, and a specific substrate for MAP kinase such as phosphorylated heat and acid stable protein regulated by insulin, or PHAS-I. The reaction is terminated by the addition of H_3PO_4 and samples are transferred to ice. An aliquot is spotted onto Whatman P81 chromatography paper, which retains the phosphorylated protein. The chromatography paper is washed and counted for ^{32}P in a liquid scintillation counter. Alternatively, the cell extract is incubated with gamma-^{32}P-ATP, an ATP regenerating system, and biotinylated myelin basic protein bound by streptavidin to a filter support.

The myelin basic protein is a substrate for activated MAP kinase. The phosphorylation reaction is carried out for 10 min at 30°C. The extract can then by aspirated through the filter, which retains the phosphorylated myelin basic protein. The filter is washed and counted for ^{32}P by liquid scintillation counting.

<u>Cell proliferation assay</u>

Receptor activation of the orphan receptor may lead to a mitogenic or proliferative response which can be monitored via ^{3}H-thymidine uptake. When cultured cells are incubated with ^{3}H-thymidine, the thymidine translocates into the nuclei where it is phosphorylated to thymidine triphosphate. The nucleotide triphosphate is then incorporated into the cellular DNA at a rate that is proportional to the rate of cell growth. Typically, cells are grown in culture for 1-3 days. Cells are forced into quiescence by the removal of serum for 24 hrs. A mitogenic agent is then added to the media. 24 hrs later, the cells are incubated with ^{3}H-thymidine at specific activities ranging from 1 to 10 uCi/ml for 2-6 hrs. Harvesting procedures may involve trypsinization and trapping of cells by filtration over GF/C filters with or without a prior incubation in TCA to extract soluble thymidine. The filters are processed with scintillant and counted for ^{3}H by liquid scintillation counting. Alternatively, adherent cells are fixed in MeOH or TCA, washed in water, and solubilized in 0.05% deoxycholate/0.1 N NaOH. The soluble extract is transferred to scintillation vials and counted for ^{3}H by liquid scintillation counting.

Alternatively, cell proliferation can be assayed by measuring the expression of an endogenous or heterologous gene product, expressed by the cell line used to transfect the orphan receptor, which can be detected by methods such as, but not limited to, florescence intensity, enzymatic activity, immunoreactivity, DNA hybridization, polymerase chain reaction, etc.

Promiscuous second messenger assays

It is not possible to predict, a priori and based solely upon the GPCR sequence, which of the cell's many different signaling pathways any given orphan receptor will naturally use. It is possible, however, to coax receptors of different functional classes to signal through a pre-selected pathway through the use of promiscuous G_α subunits. For example, by providing a cell based receptor assay system with an endogenously supplied promiscuous G_α subunit such as $G_{\alpha 15}$ or $G_{\alpha 16}$ or a chimeric G_α subunit such as $G_{\alpha qz}$, a GPCR, which might normally prefer to couple through a specific signaling pathway (e.g., G_s, G_i, G_q, G_0, etc.), can be made to couple through the pathway defined by the promiscuous G_α subunit and upon agonist activation produce the second messenger associated with that subunit's pathway. In the case of $G_{\alpha 15}$, $G_{\alpha 16}$ and/or $G_{\alpha qz}$ this would involve activation of the G_q pathway and production of the second messenger IP_3. Through the use of similar strategies and tools, it is possible to bias receptor signaling through pathways producing other second messengers such as Ca^{++}, cAMP, and K^+ currents, for example (Milligan, 1999).

It follows that the promiscuous interaction of the

exogenously supplied G_α subunit with the orphan receptor alleviates the need to carry out a different assay for each possible signaling pathway and increases the chances of detecting a functional signal upon receptor activation.

Methods for recording currents in Xenopus oocytes

Oocytes are harvested from *Xenopus laevis* and injected with mRNA transcripts as previously described (Quick and Lester, 1994; Smith et al.,1997). The test orphan receptor of this invention and Gα subunit RNA transcripts are synthesized using the T7 polymerase ("Message Machine," Ambion) from linearized plasmids or PCR products containing the complete coding region of the genes. Oocytes are injected with 10 ng synthetic receptor RNA and incubated for 3-8 days at 17 degrees. Three to eight hours prior to recording, oocytes are injected with 500 pg promiscuous Gα subunits mRNA in order to observe coupling to Ca^{++} activated Cl^- currents. Dual electrode voltage clamp (Axon Instruments Inc.) is performed using 3 M KCl-filled glass microelectrodes having resistances of 1-2 MOhm. Unless otherwise specified, oocytes are voltage clamped at a holding potential of -80 mV. During recordings, oocytes are bathed in continuously flowing (1-3 ml/min) medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 (ND96). Drugs are applied either by local perfusion from a 10 µl glass capillary tube fixed at a distance of 0.5 mm from the oocyte, or by switching from a series of gravity fed perfusion lines.

Other oocytes may be injected with a mixture of

orphan receptor mRNAs and synthetic mRNA encoding the genes for G-protein-activated inward rectifier channels (GIRK1 and GIRK4, U.S. Patent Nos. 5,734,021 and 5,728,535 or GIRK1 and GIRK2) or any other appropriate combinations (see, e.g., Inanobe et al., 1999). Genes encoding G-protein inwardly rectifying K^+ (GIRK) channels 1, 2 and 4 (GIRK1, GIRK2, and GIRK4) may be obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995 and 1995b) to derive appropriate 5′ and 3′ primers. Human heart or brain cDNA may be used as template together with appropriate primers.

Heterologous expression of GPCRs in *Xenopus* oocytes has been widely used to determine the identity of signaling pathways activated by agonist stimulation (Gundersen et al., 1983; Takahashi et al., 1987). Activation of the phospholipase C (PLC) pathway is assayed by applying test compound in ND96 solution to oocytes previously injected with mRNA for the mammalian orphan receptor (with or without promiscuous G proteins) and observing inward currents at a holding potential of -80 mV. The appearance of currents that reverse at -25 mV and display other properties of the Ca^{++}-activated Cl^- (chloride) channel is indicative of mammalian receptor-activation of PLC and release of IP3 and intracellular Ca^{++}. Such activity is exhibited by GPCRs that couple to G_q or G_{11}.

Measurement of inwardly rectifying K^+ (potassium) channel (GIRK) activity may be monitored in oocytes that have been co-injected with mRNAs encoding the mammalian orphan receptor plus GIRK subunits. GIRK

gene products co-assemble to form a G-protein activated potassium channel known to be activated (i.e., stimulated) by a number of GPCRs that couple to G_i or G_o (Kubo et al., 1993; Dascal et al., 1993). Oocytes expressing the mammalian orphan receptor plus the GIRK subunits are tested for test compound responsivity by measuring K^+ currents in elevated K^+ solution containing 49 mM K^+.

This invention further provides an antibody capable of binding to a mammalian orphan receptor encoded by a nucleic acid encoding a mammalian orphan receptor. In one embodiment, the mammalian orphan receptor is a human orphan receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian orphan receptor. In one embodiment, the antibody is a monoclonal antibody or antisera.

This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian orphan receptor, wherein the probe has a sequence corresponding to a unique sequence present within one of the two strands of the nucleic acid encoding the mammalian orphan receptor and is contained in plasmid pEXJ.T3T7-hSNORF68-f (Patent Deposit Designation No. PTA_____). This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian orphan receptor, wherein the probe has a sequence corresponding to a unique sequence present within (a) the nucleic acid sequence shown in Figure 1A-1C (SEQ ID NO: 1) or (b) the reverse complement thereto. In

one embodiment, the nucleic acid is DNA. In another embodiment, the nucleic acid is RNA.

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

Methods of preparing and employing antisense oligonucleotides, antibodies, nucleic acid probes and transgenic animals directed to the orphan SNORF68 receptor are well known in the art. (See, for example, U.S. Patent Nos. 5,053,337; 5,155,218; 5,360,735; 5,472,866; 5,476,782; 5,516,653; 5,545,549; 5,556,753; 5,595,880; 5,602,024; 5,639,652; 5,652,113; 5,661,024; 5,766,879; 5,786,155; and 5,786,157, the disclosures of which are hereby incorporated by reference in their entireties into this application.)

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